Toxicology Research Laboratory

UIC The University of Illinois at Chicago

Department of Pharmacology (M/C 868)
1940 W. Taylor St.
Chicago, Illinois 60612-7353

Title Page

Study Report for Task Order No. UIC-7

IN VITRO MUTAGENICITY TESTING OF WR242511 TARTRATE

Sponsor: US Army Medical Materiel Development Activity

Contract Number: DAMD17-92-C-2001

Test Article: WR242511 Tartrate

Principal Investigator

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Richard H. C. San, Ph.D. (Microbiological Associates, Inc.)

Completion of Testing Date

April 29, 1994

Performing Laboratory

Microbiological Assoicates, Inc. - Subcontract
Toxicology Research Laboratory (TRL)
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WR242511 Tartrate was tested for *in vitro* mutagenic potential. The Ames Test and the Mouse Lymphoma Assay were utilized to assess potential point mutations, whereas the ability to induce chromosome aberrations was tested in Chinese Hamster Ovary cells. WR242511 Tartrate was negative in the three assays utilized.

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IN VITRO MUTAGENICITY
TESTING OF WR242511 TARTRATE

Sponsor: US Army Medical Materiel
Development Activity
Fort Detrick
Frederick, MD 21701-5014

Representative: Dr. George J. Schieferstein

Test Article: WR242511 Tartrate

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.
9900 Blackwell Road
Rockville, MD 20850

as a subcontract to:

TOXICOLOGY RESEARCH LABORATORY (TRL)
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Barry S. Levine

Barry S. Levine, D.Sc., D.A.B.T.
Principal Investigator

7/18/94
Date

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1. SUMMARY

WR242511 Tartrate was tested for point mutations and chromosomal aberrations in three *in vitro* mutagenicity tests. WR242511 Tartrate was shown to be negative in the three assays utilized.

2. INTRODUCTION

The purpose of this project was to study the *in vitro* mutagenic potential of WR242511 Tartrate using the following assay systems.

1. Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

   This test evaluates the mutagenic potential of a test article (and/or its metabolites) by measuring its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of rat hepatic microsomal enzymes.

2. L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

   This assay tests for specific locus mutations at the thymidine kinase (TK) locus of cultured L5178Y TK+/- mouse lymphoma cells in the presence and absence of rat hepatic microsomal enzymes.

3. Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

   This assay evaluates clastagenic potential based upon the ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

The studies reported herein were conducted at Microbiological Associates, Inc., and the individual study reports are contained in Appendices 1 - 3.

3. RESULTS AND DISCUSSION

The results of the three assays to assess the *in vitro* mutagenicity of WR242511 Tartrate are summarized in Table 1. WR242511 Tartrate was shown to be negative in the Ames Salmonella Test, the Mouse Lymphoma Assay and the Chromosome Abberations Test using CHO cells.

4. CONCLUSION AND RECOMMENDATIONS

On the basis of the present studies, WR242511 Tartrate did not demonstrate mutagenic activity *in vitro*. As such, *in vivo* mutagenicity tests do not appear to be necessary.

Table 1

IN VITRO MUTAGENICITY TESTING OF WR242511 TARTRATE

Summary of Results

Assay	Doses Tested (ug base/ml)*	Results
Ames test		
-S9	0.33 - 33	-
+S9	33 - 333	-
Mouse lymphoma assay		
-S9	0.5 - 3.0	-
+S9	0.025 - 0.5	-
Chromosal aberrations in CHO cells test		
-S9	0.18 - 1.44	-
+S9	0.6 - 4.8	-

S9 = Metabolic Activation System (rodent liver 9000 g fraction)
- = Negative

APPENDIX 1

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION
MUTAGENICITY ASSAY (AMES TEST) REPORT

FINAL REPORT

Study Title

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Test Article

WR242511 Tartrate

Sponsor Project Number

UIC-7

Authors

Richard H.C. San, Ph.D.
Michelle L. Klug, B.S.

Study Completion Date

09/21/93

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

Laboratory Study Number

TD146.501

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Deptartment of Pharmacology Box 6998
Chicago, Il 60680

STATEMENT OF COMPLIANCE

The *Salmonella*/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test) of test article **WR242511 Tartrate** was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 792 and 40 CFR 160, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Richard H.C. San, Ph.D.
Study Director

9/21/93
Date

QUALITY ASSURANCE STATEMENT

Study Title: SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Study Number: TD146.501

Study Director: Richard H.C. San, Ph.D.

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 06/04/93 - 06/04/93, TO STUDY DIR 06/04/93, TO MGMT 06/04/93
PHASES: PROTOCOL REVIEW

INSPECT ON 07/01/93 - 07/01/93, TO STUDY DIR 07/01/93, TO MGMT 07/01/93
PHASES: COUNTING OF THE PLATES

INSPECT ON 07/19/93 - 07/19/93, TO STUDY DIR 07/19/93, TO MGMT 07/19/93
PHASES: DRAFT REPORT

INSPECT ON 09/21/93 - 09/21/93, TO STUDY DIR 09/21/93, TO MGMT 09/21/93
PHASES: DRAFT REPORT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nicole Kara Dinsmore — Date
Quality Assurance Unit

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Deptartment of Pharmacology Box 6998
Chicago, Il 60680

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR242511 Tartrate**

Test Article Lot No.: **DJD-08-235**

Test Article Bottle No.: **BM05816**

Test Article Mole Fraction: **71% base (Provided by Sponsor)**

Sponsor Project No.: **UIC-7**

MA Study No.: **TD146.501**

Test Article Description: **yellow powder**

Test Article Purity: **99.51% (Provided by Sponsor)**

Storage Conditions: **-20±5°C; protected from exposure to light**

Test Article Receipt: **05/11/93**

Study Initiation: **06/04/93**

Study Director: _____ 9/21/93
Richard H.C. San, Ph.D. Date

Laboratory Manager: Valentine O. Wagner, III 9/21/93
Valentine O. Wagner, III, M.S. Date

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SUMMARY

The test article, WR242511 Tartrate, was tested in the *Salmonella* Mutagenicity Assay using tester strains TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases using the plate incorporation method. The first phase, the dose range-finding study, was used to establish the dose range for the mutagenicity assay. The second phase, the mutagenicity assay, was used to evaluate the mutagenicity of the test article.

In the dose range-finding study, the maximum dose tested was 5000 μg base per plate. This dose, limited by the protocol, was delivered to the test system as a solution in dimethylsulfoxide (DMSO). The results of the dose range-finding study indicate that precipitate and toxicity were observed. Precipitate was present at ≥3333 μg base/plate and toxicity was observed at ≥33 μg base/plate without S9 activation and at ≥100 μg base/plate with S9 activation. The precipitate did not preclude accurate evaluation of the plates. The toxicity, presumably due to the toxic nature of the test article, was detected as a reduction in the revertant colony count and/or a reduction of the background bacterial lawn. Therefore, the maximum dose that was plated in the mutagenicity assay was 333 (to ensure toxicity with all tester strains) and 33 μg base per plate in the presence and absence of S9 activation, respectively, the maximum permissible dose based on the toxicity of the test article.

In the mutagenicity assay, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation prepared from Aroclor-induced rat liver. In addition, toxicity was observed at 33 μg base/plate without S9 activation and at ≥100 μg base/plate with S9 activation. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation* and Dose Range Tested (μg base/plate)									
	TA98		TA100		TA1535		TA1537		TA1538	
	Low	High	Low	High	Low	High	Low	High	Low	High
None	-		-		-		-		-	
	0.33	33	0.33	33	0.33	33	0.33	33	0.33	33
Rat	-		-		-		-		-	
	33	333	33	333	33	333	33	333	33	333

*-=negative, + =positive (maximum fold increase)

In conclusion, the results indicate that under the conditions of this study, test article **WR242511 Tartrate (MA# TD146)** did not cause a positive response in the *Salmonella*/Mammalian-Microsome Plate Incorporation Mutagenicity Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of S9 activation. This test system has been shown to detect a diverse group of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The ability to induce mutation is indicative of a chemical's genotoxic potential.

MATERIALS AND METHODS

Media and Reagent Preparation

Note: All references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System at MA.

On the day of its use, minimal top agar, containing 0.8 % agar (w/v) and 0.5 % NaCl (w/v), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 μM each. If the top agar was not used with S9 mix, 25 ml of sterile water were added for each 100 ml of minimal top agar.

Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (w/v) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (w/v) agar and supplemented with 2.5 % (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq$-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100. The microsomal enzyme mixture (S9 mix) was prepared immediately before its use and contained, 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4.

Test System

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a wide range of chemical classes.

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames *et al.* (1975). Tester strains in use at Microbiological Associates, Inc. (MA) were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Genotype of the Strains Used for Mutagen Testing
Figure 1

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537	TA1538	*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

The tester strains contain, in addition to a mutation in the histidine operon, two additional mutations that enhance their sensitivities to some mutagenic compounds. The *rfa* wall mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals, such as those containing large ring systems that would otherwise be excluded by a normal cell wall. The second mutation is a deletion in the *uvr*B gene that results in a deficient DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, tester strains containing this deletion also require the vitamin biotin for growth.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor) that further increases the sensitivities of these two strains to some mutagens. It has been suggested that this plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Frozen Permanent Stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing away approximately 1.5 ml aliquots in glass vials. Frozen Permanent Stocks were stored at ≤-70°C. Master plates were prepared by streaking each tester strain from a frozen permanent onto

minimal medium supplemented with histidine (260 μM), biotin (3 μM) and, for strains containing the R-factor, ampicillin (25 μg/ml). Master plates were stored at 4$\pm$2°C.

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 ml of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37$\pm$2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 1-2x10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

On the day of their use in the mutagenicity assay, all tester strain cultures were checked for the correct genotype. The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. The presence of the *uvr*B deletion was confirmed by demonstration of sensitivity to ultraviolet light. The presence of the pKM101 plasmid was confirmed by demonstration of resistance to ampicillin. Spontaneous reversion frequencies in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

The test system was exposed to the test article via the plate incorporation methodology described by Ames *et al.* (1975) and Maron and Ames (1983). This methodology has been shown to detect many classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The assay was performed in two phases. The first phase, the dose-range finding study, was used to establish the dose-range over which the test article would be assayed. Ten dose levels of the test article were plated, one plate per dose, with an overnight culture of TA100 on selective minimal agar in both the presence and absence of rat liver S9 activation. The second phase, the mutagenicity assay, was used to evaluate the mutagenicity. A minimum of five dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in triplicate.

Test and Control Articles

The test article, WR242511 Tartrate, was received by Microbiological Associates, Inc. on 05/11/93 and was assigned the code number TD146. The test article was characterized by the Sponsor as a yellow solid that should be stored at -20 to -15°C,

ambient humidity and protected from light (stored in an amber bottle) with no expiration date provided. Upon receipt, the test article was described as a yellow powder and was stored at -20±5°C, protected from exposure to light. The vehicle used to deliver WR242511 Tartrate to the test system was dimethylsulfoxide (DMSO), (CAS# 67-68-5), certified A.C.S., purchased from Fisher Scientific Company. The dosing solutions were adjusted to compensate for the mole fraction (71% base) of the test article. The dosing solutions were not adjusted to compensate for the purity of the test article. Aliquots of dosing solution preparations were returned to the Sponsor for chemical analysis.

Positive controls plated concurrently with the assay are listed in Figure 2.

Positive Controls
Figure 2

Strain	Activation	Positive Control	Concentration (μg/plate)
TA98	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1537	+	2-aminoanthracene	1.0
	-	9-aminoacridine	75
TA1538	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0

Source and Grade

9-aminoacridine (CAS #134-50-9), Aldrich Chemical Co., 98% pure
2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98 % pure
sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade

To determine the sterility of the test article, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay. To determine the sterility of the S9 mix, a 0.5 ml aliquot was plated on selective agar.

Plating and Scoring Procedures

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in Microbiological Associates, Inc.'s Microbial Mutagenesis Standard Operating Procedures.

Test article dilutions were prepared immediately before use. In the absence of S9 mix, 100 μl of tester strain and 50 μl of vehicle or test article were added to 2.5 ml of molten selective top agar at 45$\pm$2°C. When S9 mix was required, 500 μl of S9 mix, 100 μl of tester strain and 50 μl of vehicle or test article were added to 2.0 ml of molten selective top agar at 45$\pm$2°C. When plating the positive controls, the test article aliquot was replaced by a 50 μl aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for approximately 48 hours at 37$\pm$2°C. Plates that were not counted immediately following the 48 hour incubation period were stored at 4$\pm$2°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. This toxicity was scored relative to the vehicle control plate, using the criteria and codes that appear in Figure 3. Revertant colonies for a given tester strain and activation condition, except for the positive controls, were counted either entirely by an automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated. The results of these calculations are presented on the individual strain data forms.

Criteria for a Valid Test and Evaluation of Results

The following criteria must be met for the mutagenicity assay to be considered valid. All tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*), the deletion of the *uvr*B gene and the characteristic mean number of spontaneous revertants in the vehicle control as shown below:

TA98	10	-	50
TA100	80	-	240
TA1535	5	-	45
TA1537	3	-	21
TA1538	5	-	35

Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.6x10^9$ cells/ml. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn.

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA1535, TA1537 and TA1538 will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value. Data sets for strains TA98 and TA100 will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Archives

All experimental records (raw data and appropriate reports) of the study are maintained in the Microbiological Associates, Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850. The Director of the Quality Assurance Unit is responsible for maintaining the archives.

Bacterial Background Lawn Evaluation Code
Figure 3

The condition of the background bacterial lawn is evaluated, first macroscopically and then microscopically (using a dissecting microscope). Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn. The evaluation is recorded using the code described below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by a lack of microcolony lawn over up to 80% of the plate and an increase in the size of the microcolonies compared to the vehicle control plate. This increase in size is visible to the unaided eye.
5	Absent	Distinguished by a lack of microcolony background lawn over 80% of the plate.
5	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
SP	Slight Precipitate	Distinguished by a noticeable, either macro or microscopically, precipitate on the plate, however, the precipitate does not influence automated counting of the plate. If any precipitate particles are detected by the automated colony counter, the total number of particles per plate must not exceed 10% of the revertant colony count (e.g., $\leq$3 particles on a plate with 30 revertants.)
MP	Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, that influences automated counting and thus requires the plate to be hand counted. The number of precipitate particles detected by the automated colony counter must exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)
HP	Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.
Thus, 3MP would indicate a plate observed to have a moderately reduced background lawn with a marked amount of precipitate that required the plate to be counted manually.		

RESULTS AND DISCUSSION

Dose Range-Finding Study

The results of the dose range-finding study are presented in Table 1. The maximum dose tested was 5000 μg base per plate. This dose was delivered to the test system as a solution in dimethylsulfoxide (DMSO) using a plating aliquot of 50 μl.

The results of the dose range-finding study indicate that precipitate and toxicity were observed. Precipitate was present at $\geq$3333 μg base/plate and toxicity was observed at $\geq$33 μg base/plate without S9 activation and at $\geq$100 μg base/plate with S9 activation. The precipitate did not preclude accurate evaluation of the plates. The toxicity, presumably due to the toxic nature of the test article, was detected as a reduction in the revertant colony count and/or a reduction of the background bacterial lawn. Therefore, the maximum dose that was plated in the mutagenicity assay was 333 and 33 μg base per plate in the presence and absence of S9 activation, respectively, the maximum permissible dose based on the toxicity of the test article. The selection of the dose level at 333 μg base per plate was to ensure toxicity with all tester strains in case the tester strains differ in their sensitivity.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 2 through 12. These data were generated in Experiment TD146-B1. No precipitate but toxicity was observed at 33 μg base/plate without S9 avtivation and at $\geq$100 μg base/plate with S9 activation.

In Experiment TD146-B1, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation.

All criteria for a valid study were met as described in the protocol.

CONCLUSION

The results of the *Salmonella*/Mammalian-Microsome Plate Incorporation Mutagenicity Assay indicate that under the conditions of this study, **Toxicology Research Laboratoratory, University of Illinois at Chicago's** test article **WR242511 Tartrate (MA# TD146)** did not cause a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

Salmonella Mutagenicity Assay

Dose Range-Finding Study

Table 1

Test Article Id : WR242511 Tartrate
Study Number : TD146.501
Experiment No. : A1
Date Plated : 06/08/93
Counted by : machine
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl

Test Article Concentration µg base/plate	TA100 With S9 Activation Revertants per plate	TA100 With S9 Activation Background Code[a]	TA100 Without Activation Revertants per plate	TA100 Without Activation Background Code[a]
Vehicle	176	1	156	1
6.7	147	1	129	1
10	165	1	102	2
33	125	1	1[b]	4
67	168	1	0[b]	5
100	124	3	0[b]	5
333[b]	0	5	0	5
667[b]	0	5	0	5
1000[b]	0	5	0	5
3333[b]	0	5SP	0	5SP
5000[b]	0	5SP	0	5SP

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 2

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA98 Cells Seeded : 1.2 X 10^8
Liver Microsomes : None Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	23	1		
	03	25	1	20	6
0.33	01	16	1		
	02	27	1		
	03	21	1	21	6
1.0	01	28	1		
	02	25	1		
	03	20	1	24	4
3.3	01	19	1		
	02	18	1		
	03	25	1	21	4
10	01	20	1		
	02	15	1		
	03	26	1	20	6
33	01	0	4		
	02	2	4		
	03	3	4	2	2
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	355	1		
	02	319	1		
	03	344	1	339	18

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA98 Cells Seeded : 1.2 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	26	1		
	02	27	1		
	03	34	1	29	4
3.3	01	28	1		
	02	19	1		
	03	31	1	26	6
10	01	36	1		
	02	31	1		
	03	23	1	30	7
33	01	28	1		
	02	34	1		
	03	29	1	30	3
100	01	3	4		
	02	2	4		
	03	0	4	2	2
333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	2232	1		
	02	2103	1		
	03	2201	1	2179	67

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 4

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA100 Cells Seeded : 0.8 X 10^8
Liver Microsomes : None Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : machine

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	94	1		
	02	104	1		
	03	121	1	106	14
0.33	01	125	1		
	02	135	1		
	03	125	1	128	6
1.0	01	129	1		
	02	148	1		
	03	147	1	141	11
3.3	01	142	1		
	02	129	1		
	03	135	1	135	7
10	01	137	1		
	02	135	2		
	03	99	2	124	21
33	01	14	4		
	02	9	4		
	03	8	4	10	3
Positive Control sodium azide 1.0 µg per plate					
	01	550	1		
	02	575	1		
	03	568	1	564	13

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

Salmonella Mutagenicity Assay

Table 5

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA100 Cells Seeded : 0.8 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : machine

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	146	1		
	02	149	1		
	03	142	1	146	4
3.3	01	209	1		
	02	151	1		
	03	148	1	169	34
10	01	179	1		
	02	163	1		
	03	198	1	180	18
33	01	241	1		
	02	178	1		
	03	173	1	197	38
100	01	13	4		
	02	9	4		
	03	16	4	13	4
333[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	1961	1		
	02	1867	1		
	03	1834	1	1887	66

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 6

Test Article Id	: WR242511 Tartrate		
Study Number	: TD146.501	Experiment No	: B1
Strain	: TA1535	Cells Seeded	: 1.5 X 10^8
Liver Microsomes	: None	Date Plated	: 06/26/93
Vehicle	: dimethylsulfoxide (DMSO)		
Plating Aliquot	: 50 μl	Counted by	: hand

Concentration μg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	7	1		
	03	15	1	12	4
0.33	01	15	1		
	02	8	1		
	03	11	1	11	4
1.0	01	14	1		
	02	12	1		
	03	9	1	12	3
3.3	01	13	1		
	02	10	1		
	03	11	1	11	2
10	01	15	1		
	02	13	1		
	03	14	1	14	1
33	01	2	4		
	02	1	4		
	03	1	4	1	1
Positive Control sodium azide 1.0 μg per plate[b]					
	01	344	1		
	02	411	1		
	03	420	1	392	42

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 7

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA1535 Cells Seeded : 1.5 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	17	1		
	02	10	1		
	03	11	1	13	4
3.3	01	19	1		
	02	14	1		
	03	14	1	16	3
10	01	13	1		
	02	9	1		
	03	7	1	10	3
33	01	16	1		
	02	12	1		
	03	10	1	13	3
100	01	3	3		
	02	3	3		
	03	2	3	3	1
333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	145	1		
	02	153	1		
	03	136	1	145	9

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 8

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA1537 Cells Seeded : 1.0 X 10^8
Liver Microsomes : None Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	9	1		
	02	8	1		
	03	10	1	9	1
0.33	01	10	1		
	02	6	1		
	03	3	1	6	4
1.0	01	8	1		
	02	11	1		
	03	5	1	8	3
3.3	01	7	1		
	02	7	1		
	03	4	1	6	2
10	01	9	1		
	02	4	1		
	03	6	1	6	3
33	01	0	4		
	02	0	4		
	03	0	4	0	0
Positive Control 9-aminoacridine 75 µg per plate[b]					
	01	562	1		
	02	557	1		
	03	405	1	508	89

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 9

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA1537 Cells Seeded : 1.0 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	6	1		
	02	9	1		
	03	8	1	8	2
3.3	01	7	1		
	02	7	1		
	03	2	1	5	3
10	01	8	1		
	02	14	1		
	03	10	1	11	3
33	01	13	1		
	02	12	1		
	03	14	1	13	1
100	01	0	4		
	02	2	4		
	03	0	5	1	1
333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	210	1		
	02	236	1		
	03	212	1	219	14

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 10

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA1538 Cells Seeded : 4.2 X 10^8
Liver Microsomes : None Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	10	1		
	02	6	1		
	03	4	1	7	3
0.33	01	8	1		
	02	10	1		
	03	7	1	8	2
1.0	01	4	1		
	02	11	1		
	03	8	1	8	4
3.3	01	10	1		
	02	13	1		
	03	7	1	10	3
10	01	12	1		
	02	9	1		
	03	6	1	9	3
33	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	402	1		
	02	445	1		
	03	433	1	427	22

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 11

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1
Strain : TA1538 Cells Seeded : 4.2 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 06/26/93
Vehicle : dimethylsulfoxide (DMSO)
Plating Aliquot : 50 µl Counted by : hand

Concentration µg base/plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	14	1		
	03	13	1	13	1
3.3	01	11	1		
	02	17	1		
	03	14	1	14	3
10	01	12	1		
	02	13	1		
	03	10	1	12	2
33	01	16	1		
	02	11	1		
	03	12	1	13	3
100	01	2	4		
	02	1	4		
	03	3	4	2	1
333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	2317	1		
	02	2382	1		
	03	2190	1	2296	98

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay
Summary of Results

Table 12

Test Article Id : WR242511 Tartrate
Study Number : TD146.501 Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (μg base)	TA98	TA100	TA1535	TA1537	TA1538
0.0	20 ± 6	106 ± 14	12 ± 4	9 ± 1	7 ± 3
0.33	21 ± 6	128 ± 6	11 ± 4	6 ± 4	8 ± 2
1.0	24 ± 4	141 ± 11	12 ± 3	8 ± 3	8 ± 4
3.3	21 ± 4	135 ± 7	11 ± 2	6 ± 2	10 ± 3
10	20 ± 6	124 ± 21	14 ± 1	6 ± 3	9 ± 3
33	2 ± 2	10 ± 3	1 ± 1	0 ± 0	0 ± 0
Pos	339 ± 18	564 ± 13	392 ± 42	508 ± 89	427 ± 22

Liver Microsomes: Rat liver S9

Dose (μg base)	TA98	TA100	TA1535	TA1537	TA1538
0.0	29 ± 4	146 ± 4	13 ± 4	8 ± 2	13 ± 1
3.3	26 ± 6	169 ± 34	16 ± 3	5 ± 3	14 ± 3
10	30 ± 7	180 ± 18	10 ± 3	11 ± 3	12 ± 2
33	30 ± 3	197 ± 38	13 ± 3	13 ± 1	13 ± 3
100	2 ± 2	13 ± 4	3 ± 1	1 ± 1	2 ± 1
333	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pos	2179 ± 67	1887 ± 66	145 ± 9	219 ± 14	2296 ± 98

0.0 = Vehicle plating aliquot of 50 μl
Pos = Positive Control concentrations as specified in Materials and Methods section.

REFERENCES

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Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

McCann, J. and B.N. Ames (1976) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals: Discussion, Proc. Natl. Acad. Sci. USA, 73:950-954.

McCann, J., E. Choi, E. Yamasaki and B.N. Ames (1975) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals, Proc. Natl. Acad. Sci. USA, 72:5135-5139.

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

APPENDIX

Study Protocol

APPROVED

CC 6-4-93

Received by QA 6-4-93

MA Study Number: TD146.501

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of microsomal enzymes.

2.0 SPONSOR

2.1 Name: **TOXICOLOGY RESEARCH LABORATORY**

2.2 Address: University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, Il 60680

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T.

2.4 Sponsor Project #: UIC-7. This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U. S. Army Medical Research Acquisition Activity (No. DAMD17-92-C-2001). Dr. George Schieferstien, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR242511 Tartrate**

Storage: -20 to -15°C
Ambient humidity conditions
Protect from light

3.2 Controls:

Solvent:	Dimethysulfoxide (DMSO)
Negative:	Vehicle controls
Positive:	9-aminoacridine
	2-aminoanthracene
	2-nitrofluorene
	sodium azide

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic and Cellular Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 6/10/93

5.2 Proposed Experimental Completion Date: 7/9/93

5.3 Proposed Report Date: 7/16/93

6.0 TEST SYSTEM

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a wide range of chemical classes.

The tester strains to be used will be the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames *et al.* (1975).

GENOTYPE OF THE STRAINS USED FOR MUTAGEN TESTING
Figure 1

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537	TA1538	*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance their sensitivities to some mutagenic compounds. The *rfa* mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded by a normal intact cell wall. The second mutation is a deletion in the *uvr*B gene that results in a deficient

DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Finally, tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor) that further increases the sensitivities of these two strains to some mutagens. It has been suggested, that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The tester strains in use at Microbiological Associates, Inc. were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

7.1 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975). This methodology has been shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, vehicle controls and positive controls will be plated in triplicate.

7.1.1 Range-finding Assay to Select Dose Levels

Using TA100 as the indicator strain, the test article will be checked for toxicity up to a concentration of 5 mg/plate if solubility/miscibility permits. Test articles that exhibit limited solubility/miscibility will be tested for toxicity up to the maximum workable concentration attainable in the vehicle of choice. The toxicity determination will be conducted both in the presence and absence of microsomal enzymes. An aliquot from each of at least eight dilutions of the test article will be plated with an overnight TA100 culture on selective minimal agar. Toxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn. The highest concentration of test article used in the subsequent mutagenicity assay will be that which gives a detectable reduction of revertants on the selective plates and/or a thinning or disappearance of the bacterial background lawn.

If no toxicity is observed, then the highest dose level used in the mutagenicity assay will be 5 mg/plate unless:

1) The test article exhibits limited solubility or is not uniformly dispersible in the vehicle of choice.

2) The test article precipitates heavily in the top agar.

3) There is insufficient test article available to either demonstrate toxicity or achieve a maximum dose level of 5 mg/plate.

4) The Sponsor's Authorized Representative indicates an alternative top dose level.

7.1.2 Mutagenicity Assay

The test article will be tested at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98, TA100, TA1535, TA1537 and TA1538 with and without microsomal enzymes. Following an approximate 48-hour incubation at 37±2°C, revertant colonies per plate will be counted.

7.2 Exogenous Metabolic Activation

7.2.1 Liver Microsomal Enzymes - S-9 Homogenate

7.2.1.1 Species, Strain, Sex, Inducer

Liver microsomal enzymes will be prepared (under ACUC approved application Permit No. 4) from male Sprague-Dawley rats that have been injected with Aroclor 1254 at 500 mg/kg. The Aroclor will be diluted in corn oil to a concentration of 200 mg/ml. Five days after i.p. injection with the Aroclor, the rats will be sacrificed by decapitation, and their livers will be excised.

7.2.1.2 Homogenate Preparation

The preparation of the microsomal enzyme fraction will be carried out with sterile glassware and solutions at 0-4°C. Excised livers will be placed in 0.15M KCl contained in a pre-weighed beaker. After the liver is weighed, it will be transferred to another beaker containing 3 volumes of 0.15M KCl (3 ml/g of wet liver) where it will be minced with sterile scissors. The minced liver will be homogenized and centrifuged at 9000 x g for 10 minutes. The supernatant (referred to by Ames as the S-9 fraction) will be removed, and small aliquots will be distributed into freezing ampules that will be stored at ≤-70°C.

7.2.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene and 2-aminoanthracene to mutagens as described by de Serres and Shelby (1979).

7.2.2 S-9 Mix

The microsomal enzyme reaction mixture (S-9 mix) will be prepared immediately prior to its use and it will contain the following components:

H_2O	0.56 ml
1.00 M NaH_2PO_4/K_2HPO_4, pH 7.4	0.10 ml
0.20 M $MgCl_2$/0.825 M KCl	0.04 ml
0.05 M Glucose-6-phosphate	0.10 ml
0.04 M β-Nicotinamide-adenine dinucleotide phosphate	0.10 ml
S-9	0.10 ml
	1.00 ml

7.3 Tester Strain Storage

7.3.1 Frozen Permanent Stocks

Frozen Permanent Stocks are prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing at ≤-70°C approximately 1.5 ml aliquots in glass vials.

7.3.2 Master Plates

Master plates are prepared by streaking each tester strain from a frozen permanent onto minimal media appropriately supplemented with histidine (260 μM), biotin (3 μM) and when necessary for strains containing the R-factor, ampicillin (25 μg/ml). Master plates will be incubated at 37±2°C for approximately 24 hours and will be stored at 4±2°C.

7.4 Overnight Culture Preparation

Overnight cultures will be prepared by removing a colony of appropriate tester strain from the appropriate master plate and transferring it to a flask containing culture media. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

7.5 Harvesting of Cultures

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from

TD146.501

incubation at a density of approximately 1-2 x 10^9 cells/ml.

7.6 Genotype Characterization

On the day of their use in the mutagenicity assay, all tester strain cultures will be checked for the following genetic markers:

7.6.1 The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet.

7.6.2 The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light.

7.6.3 The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

7.6.4 Spontaneous reversion frequencies will be demonstrated by plating aliquots of the culture along with the appropriate vehicle on selective media.

7.7 Controls

7.7.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

POSITIVE CONTROLS
Figure 2

Strain	Activation	Positive Control	Concentration (μg/plate)
TA98	+	2-aminoanthracene	1.0
TA98	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
TA100	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
TA1535	-	sodium azide	1.0
TA1537	+	2-aminoanthracene	1.0
TA1537	-	9-aminoacridine	75
TA1538	+	2-aminoanthracene	1.0
TA1538	-	2-nitrofluorene	1.0

7.7.2 Vehicle Controls

Appropriate vehicle controls will be plated for each tester strain with and without microsomal enzymes. Vehicles compatible with this test system in order of preference include but will not be limited to: Deionized distilled grade H_2O, dimethylsulfoxide (CAS #67-68-5), ethanol (CAS #64-17-5) and acetone (CAS #67-64-1).

7.7.3 Sterility Controls

7.7.3.1 The most concentrated test article dilution will be checked for sterility.

7.7.3.2 The S-9 mix will be checked for sterility.

7.7.4 Tester Strain Titers

Each tester strain titer will be determined by plating an appropriate dilution of the overnight culture on complete agar.

7.8 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.9 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light.

7.10 Plating Procedures

Without microsomal enzymes, 100 μl of tester strain and 50 μl of vehicle or test article will be added to 2.5 ml of molten selective top agar at 45$\pm$2°C. With microsomal enzymes, 0.5 ml of S-9 mix, 100 μl of tester strain and 50 μl of vehicle or test article dilution will be added to 2.0 ml of molten selective top agar at 45$\pm$2°C. After vortexing, the mixture will be overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 hours at 37$\pm$2°C. When necessary, aliquots of other than 50 μl of test article/vehicle will be plated.

7.11 Colony Counting

The condition of the bacterial background lawn on plates in the assay will be evaluated for evidence of test article toxicity and precipitate. Evidence of

toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate.

Revertant colonies for a given tester strain and activation condition, except for the positive controls, will be counted either entirely by automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting will be counted manually.

7.12 Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation will be calculated.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

8.1.1 *rfa* Wall Mutation

To demonstrate the presence of the deep rough wall mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

8.1.2 *uvr*B Mutation

To demonstrate the presence of the *uvr*B mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light.

8.1.3 pKM101 Plasmid R-factor

To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.1.4 Characteristic Number of Spontaneous Revertants

All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	10 - 50
TA100	80 - 240
TA1535	5 - 45
TA1537	3 - 21
TA1538	5 - 35

8.1.5 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than $0.6x10^9$ cells per milliliter.

8.1.6 Positive Control Values

Each mean positive control value must exhibit at least a three fold increase over the respective mean vehicle control value for the strain.

8.2 Toxicity

A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met:

a) A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count.

b) A reduction in the background lawn.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article as specified below:

9.1 Strains TA1535, TA1537 and TA1538

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value.

9.2 Strains TA98 and TA100

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

Results of the dose range-finding study will be presented to include the number of revertants per plate and a bacterial background lawn evaluation for each dose level.

Results presented for the mutagenicity assay will include:

- the identity of the bacterial tester strains used in the assay
- details of the protocol used for preparation of the microsomal enzymes
- dose levels at which the test article was tested
- individual plate counts for all treated, positive and vehicle control plates
- mean and standard deviation for all replicate plate counts
- dose response relationship, if applicable
- evaluation of results

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to comply with all EPA and FDA test guidelines. This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes. If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor require that samples of the Test Article dosing solutions be returned? Yes. If so, indicate which doses and volume will be required.

_____ high only;

_____ high and low only;

___X___ all;

volume required ___10 ml (if possible)___.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

de Serres, F.J. and Shelby, M.D. (1979). Recommendations on data production and analysis using the *Salmonella*/ microsome mutagenicity assay. Mutation Research *64*:159-165.

14.0 APPROVAL

Barry S Levine 12/3/92
SPONSOR REPRESENTATIVE DATE

Barry S Levine
(Print or Type Name)

GS Schiefersteins 12-8-92
U.S. ARMY CONTRACTING OFFICER'S REPRESENTATIVE DATE

GEORGE J. SCHIEFERSTEIN.
(Print or Type Name)

6/4/93
STUDY DIRECTOR DATE

APPENDIX 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY REPORT

FINAL REPORT

STUDY TITLE

L5178Y TK+/- MOUSE LYMPHOMA
MUTAGENESIS ASSAY

TEST ARTICLE

WR242511 Tartrate

AUTHORS

C. Anita H. Bigger, Ph.D.
Jane J. Clarke, B.A.

STUDY COMPLETION DATE

April 26, 1994

PERFORMING LABORATORY

MICROBIOLOGICAL ASSOCIATES, INC.
9900 BLACKWELL ROAD
ROCKVILLE, MARYLAND 20850

LABORATORY STUDY NUMBER

TD146.701

SPONSOR

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, IL 60680

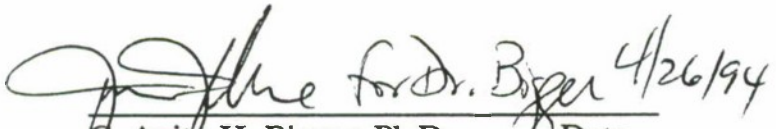
STATEMENT OF COMPLIANCE

The L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay, TD146.701, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.



C. Anita H. Bigger, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Study Number: TD146.701

Study Director: C. Anita H. Bigger, Ph.D.

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 05/25/93 - 05/25/93, TO STUDY DIR 05/27/93, TO MGMT 05/27/93
PHASES: PROTOCOL REVIEW

INSPECT ON 08/09/93 - 08/09/93, TO STUDY DIR 08/09/93, TO MGMT 08/10/93
PHASES: PREPARATION OF CULTURES

INSPECT ON 08/25/93 - 08/25/93, TO STUDY DIR 08/26/93, TO MGMT 08/26/93
PHASES: DRAFT REPORT

INSPECT ON 04/26/94 - 04/26/94, TO STUDY DIR 04/26/94, TO MGMT 04/26/94
PHASES: DRAFT REPORT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Claire L. Courtemanche — April 26, 1994

Claire L. Courtemanche
Quality Assurance Unit

Date

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, IL 60680

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **MICROBIOLOGICAL ASSOCIATES, INC.**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR242511 Tartrate**

Test Article Lot No.: **DJD-08-235**

Test Article Purity: **99.51% (provided by Sponsor)**

Test Article Mole Fraction: **71% base**

Test Article Bottle No.: **BM 05816**

Sponsor Project No.: **UIC-7**

MA Study No.: **TD146.701**

Test Article Description: **yellow powder**

Storage Conditions: **-20±5°C, protected from exposure to light**

Test Article Receipt: **May 11, 1993**

Study Initiation: **May 21, 1993**

Study Director: for Dr. Bigger 4/26/94
C. Anita H. Bigger, Ph.D. Date

Laboratory Manager: 4/26/94
Jane J. Clarke, B.A. Date

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SUMMARY

The Toxicology Research Laboratory's test article WR242511 Tartrate was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor induced rat liver S-9. The non-activated cultures selected for cloning were treated with doses of 3.0 to 0.5 μg base/ml and exhibited Total Growths from 16% to 97%. The S-9 activated cultures selected for cloning were treated with doses of 0.5 to 0.025 μg base/ml which produced from 18% to 121% Total Growths.

None of the non-activated cultures that were cloned exhibited a mutant frequency which was at least twice the mean mutant frequency of the solvent controls. A dose-dependent response was not noted in the treated cultures. None of the S-9 activated cultures that were cloned exhibited a mutant frequency which was at least twice the mean mutant frequency of the solvent controls. A dose-dependent response was not noted in the treated cultures.

The results indicate that, under the conditions of these mutagenicity tests, test article WR242511 Tartrate was negative in both the absence and presence of exogenous metabolic activation.

INTRODUCTION

Mammalian cell culture systems provide a valuable tool for assessing the genetic hazards of a variety of potentially mutagenic agents. The L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay was designed to test for specific locus mutations at the thymidine kinase (TK) locus of cultured L5178Y TK +/- mouse lymphoma cells (Clive et al., 1975).

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article or its metabolites using the L5178Y TK +/- Mouse Lymphoma Mutagenesis Assay.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR242511 Tartrate, was received by Microbiological Associates, Inc. on May 11, 1993 and was assigned the code number TD146. The test article was characterized by the Sponsor as a yellow solid, which should be stored at -20°C to -15°C under ambient humidity, protected from light in amber bottles. The mole fraction of base was 71% of the molecular weight.

Upon receipt, the test article was described as a yellow powder and was stored at -20±5°C, protected from exposure to light. At the time of dose administration, the test article was dissolved in dimethylsulfoxide, lot 933272, from Fisher Chemical Company. The Sponsor has assumed responsibility for the determination of the stability of the test article.

Ethyl methanesulfonate (EMS), lot A11212, was obtained from Eastman Kodak Chemical Company and was diluted in dimethylsulfoxide (DMSO) to stock concentrations of 50 and 25 μl/ml. 7,12-Dimethylbenz(a)anthracene (7,12-DMBA), lot 71H0269, was obtained from Eastman Kodak Chemical Company and was diluted in DMSO to stock concentrations of 500 and 250 μg/ml. DMSO, lot 933272, was obtained from Fisher Chemical Company.

MATERIALS AND METHODS

Materials

Mammalian Cells:

L5178Y cells, clone 3.7.2C, were obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, NC. Prior to using L5178Y cells for the mutation assay, the cells were cleansed of spontaneous TK -/- cells by adding a restrictive agent to the culture medium which selectively kills TK -/- mutants. Cryopreserved L5178Y cells were rapidly thawed and resuspended in flasks at a concentration of $1x10^5$ cells/ml in 100 ml of culture medium. Once normal growth was evident, the cells were cleansed of mutants. THMG (thymidine, hypoxanthine, methotrexate and glycine) was added to each flask at a concentration of 9 μg/ml thymidine, 15 μg/ml hypoxanthine, 0.3 μg/ml methotrexate, and 22.5 μg/ml glycine. The flasks were gassed with $5\pm1\%$ CO_2 in air and incubated at 37 ± 1°C in an environmental incubator shaker at 125 rpm. After approximately 24 hours, the THMG was removed by pelletizing the cells and decanting the supernatant. The cells were rinsed in 20 ml $F_{10}P$ and reinstated in culture at $3x10^4$ cells/ml in 100 ml of $F_{10}P$ with THG (3 μg/ml thymidine, 5 μg/ml hypoxanthine and 7.5 μg/ml glycine). After approximately 72 hours, the cells were ready to be used in the mutagenesis assay.

Biological Reagents:

Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F_oP), supplemented with 10% horse serum and 4mM L-glutamine ($F_{10}P$)

Trypsin, 0.1%

Trifluorothymidine (TFT), restrictive agent

Cofactors: 11.25 mg DL-Isocitric acid and 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), pH 7.0

S-9, 9000 x g supernatant of a 2:1 mixture of Aroclor-1242 and Aroclor-1254 induced Sprague-Dawley rat liver homogenate in sucrose, lot R481

Cloning medium (C.M.) containing 0.23% Agar

Chemicals:

Solvent for test article and positive controls, dimethylsulfoxide (DMSO), CAS 67-68-5

Ethyl methanesulfonate (EMS), CAS 62-50-0

Methods

The S-9 was prepared according to established procedures. Adult male Sprague-Dawley rats, 200-250 gm, were induced by a single intraperitoneal injection of a 2:1 mixture of Aroclor-1242 and Aroclor-1254 at a dosage of 500 mg/kg body weight five days prior to sacrifice. The excised tissue was rinsed three times in cold sterile 0.25 M sucrose and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 (w/v) in 0.25 M sucrose. The supernatant fraction (S-9) was collected following centrifugation at 9000 x g for 10 minutes at 4±2°C, portioned into aliquots for daily use, and stored frozen at ≤ -70°C until used. Each bulk preparation of S-9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(a)anthracene to forms mutagenic to Salmonella typhimurium TA100.

Immediately prior to use, the S-9 was mixed with the cofactors and F_oP to contain 250 ul S-9, 6.0 mg NADP, 11.25 mg DL-isocitric acid and 750 µl F_oP/ml S-9 activation mixture and kept on ice until used. The cofactor/F_oP mixture was filter sterilized and adjusted to pH 7.0 prior to the addition of S-9.

The optimal dose levels for the mutagenesis assay were selected following a preliminary toxicity test based on cell population growth relative to the solvent controls. L5178Y cells were exposed to solvent alone and nine concentrations of test article ranging from 1775 to 0.1 µg base/ml for 4 hours in the absence and presence of an exogenous source of metabolic activation. A high dose of 5000 µg base/ml could not be achieved due to lack of solubility. The osmolality of mock cultures containing the solvent only and the top dose was determined. Each tube was gassed with 5 ± 1% CO_2 in air and placed on a Bellco roller drum apparatus rotating at approximately 25 rpm. The final solvent concentration in the culture medium was 1% by volume. The test solutions were prepared under amber lights and kept in darkness during the entire exposure period. After approximately 4 hours, the test article in solution was removed by centrifuging the cells at 1000 rpm for 10 minutes and decanting the supernatant. The cells were washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml $F_{10}P$, gassed with 5 ± 1% CO_2 in air, and replaced on the roller drum apparatus. Cell population density was determined 24 and 48 hours after the initial exposure to the test article by removing a sample from each treatment tube, diluting in 0.1% trypsin, incubating at 37±1°C for 10 minutes, and counting the samples with an electronic cell counter. The cultures were adjusted to $3x10^5$ cells/ml (if the cell population exceeded $3x10^5$ cells/ml) after 24 hours only.

The mutation assay was performed according to a protocol described by Clive et al. (1975). L5178Y mouse lymphoma cells were cleansed as described previously and resuspended at a cell density of $1x10^6$ cells/ml.

Sixteen concentrations of the test article were added to empty centrifuge tubes. Two control tubes received solvent only and the positive controls were treated with EMS (0.5 and 0.25 µl/ml) and 7,12-DMBA (5 and 2.5 µg/ml). Four ml of F_oP were added to the non-activated cultures and 4 ml of S-9 reaction mixture were added to the activated

cultures. Six ml aliquots of the cell suspension were then dispensed into the tubes to yield $6x10^6$ cells/centrifuge tube. The final solvent concentration in the culture medium was 1% by volume. After the treatment period, for 4 hours at 37±1°C, the cells were washed twice with $F_{10}P$ by centrifuging the cultures at 1000 rpm for 10 minutes and decanting the supernatant. The cells were resuspended in $F_{10}P$, gassed with 5±1% CO_2 in air and placed on the roller drum apparatus at 37±1°C.

For expression of the mutant phenotype, the cultures were counted and adjusted to $3x10^5$ cells/ml (if the cell population exceeded $3x10^5$ cells/ml) at approximately 24 and 48 hours after treatment in 20 and 10 ml total volume, respectively.

For expression of the TK -/- cells, cells were placed in cloning medium (C.M.) containing 0.23% granulated agar. Two flasks per culture to be cloned were labeled with the test article concentration, activation condition, and either TFT or V.C. (viable count). Each flask was prewarmed to 37±1°C, filled with 100 ml C.M., and placed in an incubator shaker at 37±1°C until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were resuspended in 2 ml of C.M. from the corresponding TFT flask. A $2x10^{-4}$ dilution was carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml of $F_{10}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1.0 ml of stock solution of TFT was added to the TFT flask (final concentration of 3 μg/ml) and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37±1°C. After 15 minutes, the flasks were removed one at a time, and 33 ml of the cell suspension was pipetted into each of three appropriately labeled petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 20 minutes. The plates were removed and incubated at 37±1°C in a humidified 5±1% CO_2 atmosphere for 10-12 days.

Controls

EMS was used as the positive control in the non-activated study at two final concentrations of 0.5 and 0.25 μl/ml. 7,12-DMBA was used as the positive control in the S-9 activated study at two final concentrations of 5.0 and 2.5 μg/ml. The solvent for the test article was used as the solvent control at the same concentration (1% of total volume) as that found in the test article-treated groups.

Evaluation of Test Results

Some mathematical calculations were conducted utilizing non-rounded numbers whereas only rounded values were presented. Therefore, reported results of calculations found in the tables may differ slightly from results calculated using only rounded values.

After the incubation period, both the TFT plates and the V.C. plates were scored for the total number of colonies per plate. Three counts per plate were made on an automatic colony counter, and the median count was recorded. The mutation frequency

was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C. plates and multiplying the quotient by two hundred. This calculation yields a mutant frequency expressed as the number of mutants per 10^6 clonable cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive, equivocal or negative response according to the following criteria:

> Positive - A test article producing a concentration-related increase in mutant frequency with one or more of the doses in the 10% or greater total growth range exhibiting a mutant frequency which is two-fold greater than the background level will be considered positive.
>
> Equivocal - A test article producing no concentration-related increase in mutant frequency but any one or more of the three highest doses in the 10% or greater total growth range exhibit a mutant frequency which is two-fold greater than the background level, or if there is a concentration-related increase but no culture exhibits a two-fold increase in mutant frequency over background will be considered equivocal.
>
> Negative - A test article producing no concentration-related increase in mutant frequency and no two-fold or greater increase above the background in dose levels in the 10% or greater total growth range will be considered negative.

Criteria for Determination of a Valid Test

The mutant frequency of the positive controls must be at least twice that of the appropriate solvent control cultures. The spontaneous mutant frequency of the solvent controls must be between 20 and 100 per 10^6 surviving cells. The plating efficiency of the solvent controls must be greater than 50%.

Records

All raw data, draft and final reports are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

REFERENCES

Clive, D. and J.F.S. Spector. 1975. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research 31:17-29.

RESULTS AND DISCUSSION

The Initial Toxicity Test (Table 1) conducted on test article WR242511 Tartrate, adjusted for 71% base, indicated 100% toxicity down through 5.0 μg base/ml for both the non-activated cultures and the S-9 activated cultures. The osmolality of the solvent control was 439 mOsm/kg and the osmolality of the top dose, 1775 μg base/ml, was 427 mOsm/kg. Based on the results of the initial toxicity test, the doses chosen for the mutagenesis assay ranged from 5.0 to 0.3 μg base/ml for the non-activated and from 3.0 to 0.025 μg base/ml for the S-9 activated cultures.

The cloning data for test article WR242511 Tartrate and positive controls in the absence and presence of an exogenous metabolic activation system are presented in Tables 2 and 4. Total compound toxicity data are presented in Tables 3 and 5.

After a two-day expression period, ten doses of the non-activated and seven doses of the S-9 activated cultures were selected for cloning. The non-activated cultures that were cloned had been treated with 3.0, 2.5, 2.0, 1.5, 1.0, 0.9, 0.8, 0.7, 0.6 and 0.5 μg base/ml which produced a range in Suspension Growth of 18% to 99%. The S-9 activated cultures that were cloned had been treated with 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 and 0.025 μg base/ml which produced a range in Suspension Growth of 21% to 103%.

None of the non-activated cultures that were cloned exhibited a mutant frequency which was at least two times the mean mutant frequency of the solvent controls. The Total Growths of these cultures ranged from 16% to 97%. A dose-dependent response was not noted in the treated cultures.

None of the S-9 activated cultures that were cloned exhibited a mutant frequency which was at least two times the mean mutant frequency of the solvent controls. The Total Growths of these cultures ranged from 18% to 121%. A dose-dependent response was not noted in the treated cultures.

CONCLUSION

The solvent and positive controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, WR242511 Tartrate was found to be negative in both the absence and presence of exogenous metabolic activation.

TABLE 1

INITIAL COMPOUND TOXICITY TEST USING WR242511 Tartrate

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	% of Control[c]
WITHOUT ACTIVATION				
1775	0.106	0.129	0.0	0
1000	0.072	0.050	0.0	0
500	0.016	0.022	0.0	0
100	0.007	0.008	0.0	0
50	0.002	0.019	0.0	0
10	0.001	0.023	0.0	0
5.0	0.013	0.042	0.0	0
1.0	1.017	1.400	15.8	86
0.1	1.074	1.443	17.2	94
Solvent 1	1.080	1.431	17.2	
Solvent 2	1.079	1.618	19.4	
WITH S-9 ACTIVATION				
1775	0.060	0.025	0.0	0
1000	0.004	0.004	0.0	0
500	0.060	0.032	0.0	0
100	0.001	0.011	0.0	0
50	+	+		
10	0.209	0.107	0.0	0
5.0	0.000	0.008	0.0	0
1.0	0.344	0.251	1.0	7
0.1	0.784	1.457	12.7	93
Solvent 1	0.814	1.432	13.0	
Solvent 2	0.845	1.534	14.4	

+ - Culture Lost

[a] - Cultures containing <$0.3x10^6$ cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / $0.3x10^6$ cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

TABLE 2

CLONING DATA FOR L5178Y TK+/- MOUSE LYMPHOMA CELLS TREATED WITH WR242511 Tartrate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
3.0	15/3	2	127/3	7	24	3	16
2.5	17/3	3	131/3	12	26	5	27
2.0	11/3	2	143/3	16	15	-6	48
1.5	16/3	2	134/3	11	24	3	76
1.0	14/3	2	129/3	10	22	1	80
0.9	12/3	2	143/3	6	17	-4	90
0.8	14/3	3	139/3	9	20	-1	93
0.7	19/3	3	132/3	9	29	8	90
0.6	14/3	2	148/3	4	19	-2	97
0.5	10/3	2	122/3	11	16	-5	81
Solvent 1	16/3	2	150/3	15	21		
Solvent 2	16/3	3	151/3	1	21		
Solvent 3	13/3	1	155/3	6	17		
Solvent 4	16/3	2	124/3	9	26		
Mean Solvent Mutant Frequency= 21							
Positive Control - Ethyl Methanesulfonate (µl/ml)							
0.50	265/3	31	91/3	14	582	562	41
0.25	185/3	14	128/3	17	289	269	78
Solvent 1	11/3	2	135/3	10	16		
Solvent 2	16/3	4	132/3	7	24		
Mean Solvent Mutant Frequency= 20							

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = (Average # TFT colonies / average # VC colonies) x 200

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 3

TOTAL COMPOUND TOXICITY DATA FOR L5178Y TK+/- MOUSE LYMPHOMA CELLS
TREATED WITH WR242511 Tartrate
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
4.0	0.052	0.158	0.0	0	++		
3.5	0.107	0.285	0.0	0	++		
3.0	0.228	0.777	2.6	18	127	88	16
2.5	0.372	1.031	4.3	30	131	90	27
2.0	0.413	1.490	6.8	48	143	99	48
1.5	0.869	1.228	11.9	83	134	92	76
1.0	0.934	1.239	12.9	90	129	89	80
0.9	1.002	1.168	13.0	91	143	99	90
0.8	0.981	1.278	13.9	97	139	96	93
0.7	1.013	1.253	14.1	99	132	91	90
0.6	1.033	1.182	13.6	95	148	102	97
0.5	1.012	1.236	13.9	97	122	84	81
Solvent 1	0.959	1.321	14.1		150		
Solvent 2	1.063	1.205	14.2		151		
Solvent 3	0.984	1.313	14.4		155		
Solvent 4	1.054	1.238	14.5		124		
Positive Control - Ethyl Methanesulfonate (µl/ml)							
0.50	0.726	1.089	8.8	61	91	68	41
0.25	0.828	1.262	11.6	81	128	96	78
Solvent 1	1.055	1.221	14.3		135		
Solvent 2	1.030	1.270	14.5		132		

++ - Too Toxic To Clone

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 4

CLONING DATA FOR L5178Y TK+/- MOUSE LYMPHOMA CELLS
TREATED WITH WR242511 Tartrate
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
0.5	11/3	2	105/3	7	21	-6	18
0.4	14/3	2	114/3	4	25	-2	28
0.3	16/3	4	131/3	11	24	-3	58
0.2	15/3	2	129/3	7	23	-4	87
0.1	24/3	5	143/3	5	34	7	114
0.05	18/3	5	144/3	3	25	-2	121
0.025	20/3	3	125/3	9	32	5	104
Solvent 1	18/3	5	129/3	17	28		
Solvent 2	13/3	3	115/3	6	23		
Solvent 3	15/3	3	132/3	4	23		
Solvent 4	19/3	5	117/3	7	32		
Mean Solvent Mutant Frequency= 27							
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)							
5.0	19/3	5	7/3	1	543	512	0
2.5	128/3	14	98/3	11	261	230	46
Solvent 1	19/3	4	120/3	7	32		
Solvent 2	16/3	2	110/3	1	29		
Mean Solvent Mutant Frequency= 31							

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = (Average # TFT colonies / average # VC colonies) x 200

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 5

TOTAL COMPOUND TOXICITY DATA FOR L5178Y TK+/- MOUSE LYMPHOMA CELLS
TREATED WITH WR242511 Tartrate
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
0.7	0.360	0.255	1.0	9	++		
0.6	0.391	0.274	1.2	10	++		
0.5	0.431	0.505	2.4	21	105	85	18
0.4	0.493	0.648	3.5	30	114	92	28
0.3	0.626	0.911	6.3	55	131	106	58
0.2	0.682	1.255	9.5	83	129	105	87
0.1	0.675	1.504	11.3	98	143	116	114
0.05	0.772	1.386	11.9	103	144	117	121
0.025	0.785	1.352	11.8	103	125	101	104
Solvent 1	0.788	1.256	11.0		129		
Solvent 2	0.698	1.462	11.3		115		
Solvent 3	0.773	1.427	12.3		132		
Solvent 4	0.757	1.345	11.3		117		
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)							
5.0	0.302	0.305	1.0	8	7	6	0
2.5	0.541	1.087	6.5	54	98	85	46
Solvent 1	0.793	1.393	12.3		120		
Solvent 2	0.775	1.349	11.6		110		

++ - Too Toxic To Clone

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

APPENDIX

Study Protocol

APPROVED
CC 5-21-93

5/25/93

MA Study Number: TD146.701

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article (or its metabolites) using the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay.

2.0 SPONSOR

2.1 Name: **TOXICOLOGY RESEARCH LABORATORY**

2.2 Address: University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, Il 60680

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T.

2.4 Sponsor Project #: UIC-7. This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR242511 Tartrate**

Storage: -20 to -15°C
Ambient humidity conditions
Protect from light

3.2 Controls:
Solvent: Dimethysulfoxide (DMSO)
Negative: Test Article Vehicle
Positive: Ethylmethanesulfonate (EMS)
7,12-dimethylbenz(a)anthracene (DMBA)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Genetic and Cellular Toxicology Division Microbiological Associates, Inc.
4.2	Address:	9900 Blackwell Road Rockville, MD 20850
4.3	Study Director:	C. Anita H. Bigger, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 6/15/93

5.2 Proposed Experimental Completion Date: 9/6/93

5.3 Proposed Report Date: 9/10/93

6.0 TEST SYSTEM

L5178Y TK+/- mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

6.1 Source

The L5178Y TK+/- mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutagenesis assay consists of a solubility or miscibility test to select a suitable solvent, followed by a preliminary toxicity test and then the mutagenesis test. The toxicity test is performed with and without S-9 activation to determine the optimal dose levels at which to assay the test article. The mutagenesis test is then conducted with and without S-9 activation at the desired doses. Following the addition of the test article to the cultures, they are incubated for a 4-hour exposure period. The test article is then removed and the cultures are incubated for a 2-day expression period in order that induced mutations be expressed. Cell population adjustments are made at 24 and 48 hours and those cultures exhibiting 0% to 90% growth inhibition are cloned in restrictive medium in soft agar on the second day. After a 10 to 12-day incubation period, the plates are scored and the mutant frequencies are calculated. A test article is designated as having induced a mutagenic response when a mutant frequency significantly higher than that of the

control cultures is observed and the response is dose-dependent. The diameters of the mutant colonies are also determined when there is a significant increase in mutant frequency.

7.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, and 0.1 ug/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay, so that the highest concentration exhibits approximately 100% growth inhibition, without a change in osmolality, and the survival in the lowest concentration approximates that of the negative control. The test article will be solubilized and 15 serial dilutions will be carried out to produce 16 dose levels.

7.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.3 Controls

7.3.1 Solvent Control

The vehicle for the test article will be used as the solvent control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled water or F_0P, dimethysulfoxide (DMSO), ethanol and acetone.

7.3.2 Positive Controls

Two concentrations (0.5 ul/ml and 0.25 ul/ml) of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations (5.0 ug/ml and 2.5 ug/ml) of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.4 Exogenous Metabolic Activation

7.4.1 S-9

The metabolic activation system will be a 9000 x g supernatant fraction

of rat liver homogenate (S-9) (prepared under ACUC approved application Permit No. 04) with added cofactors. The source of S-9 will be adult male Sprague-Dawley rats induced by a single injection of a 2:1 mixture of Aroclor 1242 and Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S-9 will be stored frozen at $\leq$-70°C until used.

7.4.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

DL-isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The DL-isocitric acid and NADP will be dissolved in cold Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic (F_0P) and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate the cofactor mix will be filter sterilized.

7.5 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and without S-9 activation will be conducted.

The test article will be solubilized and diluted in the compatible solvent. The dilutions of test article and the solvent controls will be added to each appropriately labeled polypropylene centrifuge tube in amounts at which the final solvent concentration is non-toxic to the cell suspension. Four milliliters of medium or S-9 activation mixture will be added to the tubes depending on whether or not they receive activation. The pH of the treatment medium will be determined at this point and readjusted to neutral if necessary. The osmolality of the culture treated with the highest dose of test article will be determined by setting up a mock culture tube containing the test article dilution and 10 ml of F_0P. If the osmolality is too high, the ion content of the treatment medium will be reduced by reducing the concentration of test article. Cultures will be initiated for each activation condition by seeding each centrifuge tube per dose level and two per solvent control with 6 ml of a cell suspension from a common pool containing 1×10^6 cells/ml. Each tube will be gassed with $5 \pm 1\%$ CO_2 in air and placed on a roller drum apparatus rotating at approximately 25 rpm at 37 ± 1°C for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be

prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 rpm for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of F_0P with 10% heat-inactivated horse serum ($F_{10}P$), resuspended in 20 ml of $F_{10}P$, gassed with 5 $\pm$ 1% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37 $\pm$ 1°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter. If test article debris prevents counting by an electronic cell counter, cell concentrations will be determined by trypan blue exclusion using a hemacytometer.

7.6 Testing for Mutagenic Activity

7.6.1 Cell Preparation

Prior to use in the assay, L5178Y TK+/- cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK-/- cells. Three ml of THMG (thymidine, hypoxanthine, methotrexate and glycine) stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5 $\pm$ 1% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37 $\pm$ 1°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG (thymidine, hypoxanthine and glycine) stock solution. The cells will be ready for use after 72 hours of incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37 $\pm$ 1°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0 x 10^6 cells per ml will be prepared. If cultures are to be treated with more than 0.1 ml of test article dilution, the cell concentration may be increased.

7.6.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is non-toxic to the cell suspension. The compound will be tested with and without S-9 activation. Four milliliters of S-9 activation mixture will be added to half of the tubes and 4 ml of F_0P will be added to the other half. Any pH adjustments with HCl or NaOH will take place after the addition of F_0P to the non-activated culture tubes. The corresponding S-9 activated culture tubes will be treated with the same amount of HCl or NaOH prior to the addition of the S-9 activation mixture. Then the appropriate volume of the cell suspension will be added to yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of TK-/- colonies, at least two control tubes per activation condition will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with $5 \pm 1\%$ CO_2 in air and placed on a roller drum apparatus for 4 hours at $37 \pm 1°C$. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 rpm for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with $5 \pm 1\%$ CO_2 in air, and replaced on the roller drum apparatus at $37 \pm 1°C$.

7.6.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.6.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK-/- cells to grow. The restrictive medium used is cloning medium (C.M.) with trifluorothymidine (TFT) at 2-4 ug/ml. The C.M. will contain approximately 0.23% agar which maintains the cells in suspension and allows them to form discrete colonies of TK-/- cells. Those cultures

exhibiting approximately 0% to 90% growth inhibition will be cloned.

Two flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37 ± 1°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37 ± 1°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3 x 10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3 x 10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 rpm for 10 minutes and the supernatant will be decanted. The cells will be resuspended in 2 ml of medium from the appropriately labeled TFT flask and added to that flask.

A 2 x 10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 9 ml of $F_{10}P$, adding 1.0 ml of this to 4 ml $F_{10}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, an aliquot of the restrictive agent at 2-4 ug/ml will be added to the TFT flask, and both this flask and V.C. flask will be placed on the shaker at 125 rpm and 37 ± 1°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted evenly into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (0-6°C) for approximately 20 to 30 minutes. The plates will be removed and incubated at 37 ± 1°C in a humidified 5 ± 1% CO_2 atmosphere for 10-12 days.

7.7 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the colonies will be counted by eye. The mutant frequency per 10^6 surviving cells will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C.

plates and multiplying the quotient by two hundred. By comparing the mutant frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected. If the test article induces a positive or equivocal response, the diameters of the TFT colonies will be determined over a range of 0.2 to 1.1 mm.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 The mutant frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

8.2 The spontaneous mutation frequency of the solvent control cultures must be between 20 and 100 per 10^6 surviving cells.

8.3 The cloning efficiency of the solvent controls must be greater than 50%, that is, greater than 100 V.C. colonies average per solvent control culture.

9.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated, therefore the conclusion of the study will be based on the Study Director's evaluation and interpretation of the data.

9.1 Positive - if there is a positive dose response and one or more of the doses in the 10% or greater Total Growth range exhibit a mutant frequency which is two-fold greater than the background level. All data including that from cultures with less than 10% Total Growth will be used to establish the dose response relationship.

9.2 Equivocal - if there is no dose response but any one or more of the three highest doses with 10% or greater Total Growth exhibit a two-fold increase in mutant frequency over background, or if there is a dose response but no culture exhibits a two-fold increase in mutant frequency over background.

9.3 Negative - if there is no dose response in cultures with 10% or greater Total Growth and none of these test cultures exhibit a two-fold or greater increase in mutant frequency over background.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.1 The data from the toxicity test.

10.2 The data generated by the mutagenesis assays which includes:

10.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2 The number of TK-/- colonies per TFT plate for the test article and the controls.

10.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.4 The mutant frequency of each dose level of the test article and the controls.

10.2.5 The induced mutant frequency of each dose level of the test article and the positive controls.

10.2.6 The distribution of the mutant colony sizes for each significantly mutagenic dose level of test article and for the controls.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc. in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to follow EPA and OECD test guidelines. A confirmatory assay would have to be conducted for full compliance and duplicate cultures may be required in some cases.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes

13.0 REFERENCES

Clive, D. and Spector, J.F.S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31:17-29, 1975.

14.0 APPROVAL

Barry A Levine 12/3/92
SPONSOR REPRESENTATIVE DATE

Barry S Levine
(Print or Type Name)

GS Schieferstein 12-8-92
U.S. ARMY CONTRACTING OFFICER'S REPRESENTATIVE DATE

GEORGE J. SCHIEFERSTEIN.
(Print or Type Name)

for Dr. Anita Biggs 5/21/93
STUDY DIRECTOR DATE

APPENDIX 3

CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS REPORT

FINAL REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

WR242511 Tartrate

Authors

Donald L. Putman, Ph.D.
Patrick T. Curry, Ph.D.

Study Completion Date

April 29, 1994

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, Maryland 20850

Laboratory Study Number

TD146.337

Sponsor Study Number

UIC-7

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, Il 60680

STATEMENT OF COMPLIANCE

The cytogenetics study, Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells , TD146.337, was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Donald L. Putman, Ph.D.
Study Director

4-25-94
Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: TD146.337

Study Director: Donald L. Putman, Ph.D.

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 06/03/93 - 06/03/93, TO STUDY DIR 06/03/93, TO MGMT 06/03/93
PHASES: PROTOCOL REVIEW

INSPECT ON 06/30/93 - 06/30/93, TO STUDY DIR 06/30/93, TO MGMT 07/01/93
PHASES: PREPARATION OF S-9 MEDIUM

INSPECT ON 09/30/93 - 09/30/93, TO STUDY DIR 09/30/93, TO MGMT 10/01/93
PHASES: DRAFT REPORT

INSPECT ON 04/29/94 - 04/29/94, TO STUDY DIR 04/29/94, TO MGMT 04/29/94
PHASES: DRAFT REPORT TO FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nicole K. Dinsmore — 4/29/94

Nicole Kara Dinsmore
Quality Assurance Unit

Date

CHROMOSOME ABERRATIONS
IN CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology Box 6998

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR242511 Tartrate**

Test Article Lot No.: **DJD-08-235**

Test Article Bottle No.: **BM05816**

Test Article Purity: **99.51% (provided by Sponsor)**

Test Article Mole Fraction: **71% base (provided by Sponsor)**

Sponsor Project No.: **UIC-7**

MA Study No.: **TD146.337**

Test Article Description: **Yellow powder**

Storage Conditions: **-20 to -15°C, protected from light**

Test Article Receipt: **May 11, 93**

Study Initiation: **June 3, 1993**

Study Director: _____ 4-29-94
Donald L. Putman, Ph.D. Date

Associate Study Director: _____ 4-29-94
Patrick T. Curry. Ph.D. Date

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SUMMARY

The test article, WR242511 Tartrate, was tested in the chromosome aberration assay using Chinese hamster ovary cells. The assay was conducted at dose levels of 0.09, 0.18, 0.36, 0.72, 1.44, and 2.88 μg/ml in the absence of metabolic activation, and 0.3, 0.6, 1.2, 2.4, and 4.8 μg/ml in the presence of an Aroclor-induced S-9 metabolic activation system . Due to an observed delay in cell cycle kinetics in the toxicity study, metaphase cells were collected at 14 hours after initiation of treatment in order to assure microscopic evaluation of first-division metaphase cells. Mitotic inhibition, in the toxicity study was seen at and above dose level 4.8 μg/ml in the nonactivated and S-9 activated studies. Excessive toxicity, (i.e., extreme mitotic inhibition), precluded the evaluation of dose level 2.88 μg/ml in the nonactivated chromosome aberration study. To assure four scorable dose levels, dose levels 0.09 μg/ml and 0.3 μg/ml were also tested in the nonactivated and S-9 activated systems respectively, but were not required for microscopic analysis. No significant increase in chromosome aberrations was observed in the non-activated or S-9 activated test systems relative to the solvent control group ($p > 0.05$, Fishers exact test). WR242511 Tartrate was concluded to be negative in the chromosome aberrations assay in Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR242511 Tartrate, was received by Microbiological Associates, Inc. on May 5, 1993 and was assigned the code number D146. The test article was characterized by the Sponsor as a yellow powder that should be stored at -20 to -15°C, protected from exposure to light, without an expiration date provided.

Upon receipt, the test article was described as a yellow powder and was stored at -20±5°C protected from exposure to light. At the time of use, the test article was dissolved in DMSO, CAS 67-68-5, lot 933272, obtained from Fisher.

Triethylenemelamine (TEM), lot 80386, was obtained from Polysciences, Inc., and was dissolved and diluted in deionized, distilled water to two stock concentrations of 25 and 50 μg/ml. Cyclophosphamide (CP), lot 72H0088, was obtained from Sigma Chemical Company, and was dissolved and diluted in deionized, distilled water to two stock concentrations of 2.5 and 5 mg/ml.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. In order to assure the karyotypic stability of the cell line, cells were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination.

Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 1.4 mg nicotinamide adenine dinucleotide phosphate (NADP), 2.7 mg isocitric acid and 15 μl S-9 per milliliter medium (McCoy's 5A supplemented with 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine).

Preliminary Toxicity Assay

The toxicity test was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of test article effect on mitotic indices and cell cycle delay. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine) for the non-activated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl dosing solution of test article in solvent or solvent alone. The cells were treated for six hours in the non-activated test system. Two hours after initiation of treatment, a 50 μl aliquot of 1mM 5-bromo-2'-deoxyuridine (BrdU) was added to each flask and incubation continued as required. At completion of the 6 hour exposure period, the treatment medium was removed, the cells washed with phosphate buffered saline (PBS), refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from the initiation of BrdU treatment. In the S-9 activated system, the cells were treated for two hours after which the treatment medium was removed, the cells washed with PBS, refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from the initiation of BrdU treatment. Two hours prior to harvest by trypsinization, Colcemid was added to each flask at a final concentration of 0.1 μg/ml. Metaphase preparations were prepared and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides were evaluated for the percentage of first, second and third-plus-subsequent-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The mitotic index was determined for each treatment condition as the percentage of mitotic cells in a population of 500 cells scored.

Definitive Assay

For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium for the non-activated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture was also included.

In the non-activated study, the cells were exposed for 12 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest, the treatment medium was removed in order to avoid interference with cell collection and fixation and the cells were washed with PBS and refed with complete medium containing 0.1 μg/ml of Colcemid.

In the S-9 activated study, the cells were exposed for 2 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with PBS, refed with complete medium and returned to the incubator for an additional 10 hours. At this time, Colcemid was added to duplicate flasks for each treatment condition at a final concentration of 0.1 μg/ml and the flasks incubated for an additional two hours.

Collection of Metaphase Cells

Two hours after the addition of Colcemid, metaphase cells were harvested for both the non-activated and S-9 activated studies by trypsinization. Cells were collected approximately 14 hours after initiation of treatment. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant fluid decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a moist glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads (50 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and

exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted.

Controls

TEM was used as the positive control in the non-activated study at final concentrations of 0.25 and 0.50 μg/ml. CP was used as the positive control in the S-9 activated study at final concentrations of 25 and 50 μg/ml. The concentration of TEM and CP that resulted in 200 scorable metaphase cells was used as the the positive control in the nonactivated and S-9 activated studies, respectively. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S-9 reaction mixture was used in the untreated control.

Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for the toxicity and aberration study. The number and types of aberrations found are presented for each treatment group. The percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined was calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) was also calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at

one dose level other than the high dose with no dose response was considered equivocal.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

All raw data, draft and final reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity test based upon reduction in mitotic index after treatment relative to the solvent control (Tables 1 and 2). CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.05 μg/ml to 480 μg/ml in the absence and presence of an S-9 reaction mixture. The test article was soluble in solvent at stock concentration 48 mg/ml, but insoluble in treatment medium at test concentration 480 μg/ml. The test article was soluble in treatment medium at all other concentrations tested. The osmolality of the highest concentration tested, 480 μg/ml, was 428 mOsm/kg. Based upon the findings of the toxicity study, dose levels of 0.09, 0.18, 0.36, 0.72, and 1.44 μg/ml, in the nonactivated and 0.3, 0.6, 1.2, 2.4, and 4.8 μg/ml in the S-9 activated were selected for further study. The highest doses for both the nonactivated and the activated test systems were initially to be chosen based on at least a 50% inhibition of mitosis. The toxicity test demonstrated no toxicity at 1.44 μg/ml and complete toxicity at the next highest dose, 4.8 μg/ml. In view of this steep toxicity the high dose was chosen to be 2.88 μg/ml, but was precluded from evaluation as described below. The highest dose chosen in the S-9 activated test system demonstrated approximately an 80% inhibition. Due to an observed delay in cell cycle kinetics, the harvest times were set at 14 hours in order to assure that cells were evaluated in first division metaphase after treatment.

The activity of WR242511 Tartrate in the induction of chromosome aberrations in CHO cells when treated in the absence of an exogenous source of metabolic activation is presented by treatment flask in Table 3 and summarized by group in Table 5. The test article was soluble in solvent at stock concentration 0.288 mg/ml

and was soluble in treatment medium at dose level 2.88 μg/ml. Excessive toxicity, (i.e., extreme mitotic inhibition) precluded evaluation of dose level 2.88 μg/ml. Toxicity (mitotic inhibition) was approximately 42% at 1.44 μg/ml, the highest test concentration evaluated for structural chromosome aberrations. To assure four scorable dose levels, dose level 0.09 μg/ml was also tested but was not required for microscopic analysis. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control ($p > 0.05$, Fisher's exact test). The percentage of damaged cells in the TEM group was 26% ($p \leq 0.01$, Fisher's exact test).

The activity of WR242511 Tartrate in the induction of chromosome aberrations in CHO cells when treated in the presence of an S-9 reaction mixture is presented by treatment flask in Table 4 and summarized by group in Table 5. The test article was soluble in solvent at stock concentration 0.48 mg/ml and was soluble in treatment medium at dose level 4.8 μg/ml. Toxicity (mitotic inhibition) was approximately 59% at 4.8 μg/ml, the highest test concentration evaluated for structural chromosome aberrations. To assure four scorable dose levels, dose level 0.3 μg/ml was also tested but was not required for microscopic analysis. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control ($p > 0.05$, Fisher's exact test). The percentage of damaged cells in the CP group was 10% ($p \leq 0.01$, Fisher's exact test).

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, WR242511 Tartrate was concluded to be negative in the chromosome aberrations in Chinese hamster ovary (CHO) cells cytogenetics assay.

TABLE 1
PRELIMINARY TOXICITY TEST USING WR242511 TARTRATE IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3
DMSO	4.4		4	96	0
WR242511 Tartrate					
0.05 ug/ml	4.0	-9	4	96	0
0.14 ug/ml	5.4	23	6	94	0
0.48 ug/ml	6.0	36	5	95	0
1.44 ug/ml	6.2	41	24	76	0
4.8 ug/ml	0.0	-100	0	0	0
14.4 ug/ml	0.0	-100	0	0	0
48 ug/ml	0.0	-100	0	0	0
144 ug/ml	0.0	-100	0	0	0
480 ug/ml	0.0	-100	0	0	0

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.

TABLE 2
PRELIMINARY TOXICITY TEST USING WR242511 TARTRATE IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3
DMSO	4.2		11	89	0
WR242511 Tartrate					
0.05 ug/ml	7.4	76	12	88	0
0.14 ug/ml	5.2	24	6	94	0
0.48 ug/ml	5.0	19	5	95	0
1.44 ug/ml	4.8	14	10	90	0
4.8 ug/ml	0.8	-81	0	100	0
14.4 ug/ml	0.0	-100	0	0	0
48 ug/ml	0.0	-100	0	0	0
144 ug/ml	0.0	-100	0	0	0
480 ug/ml	0.0	-100	0	0	0

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 2 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.

TABLE 3
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR242511 TARTRATE IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
					14 Hour Harvest							
Untreated cells	A	5.2	50	4	0	0	0	0	2	0	0	0.040
	B	5.2	50	0	0	0	0	0	0	0	0	0.000
DMSO	A	7.0	50	0	0	0	0	0	0	0	0	0.000
	B	6.8	50	2	0	0	0	1	0	0	0	0.020
WR242511 Tartrate												
0.18 ug/ml	A	6.0	50	0	0	0	0	0	0	0	0	0.000
	B	5.2	50	2	0	1	0	0	0	0	0	0.020
0.36 ug/ml	A	5.0	50	2	0	0	0	1	0	0	0	0.020
	B	4.4	50	2	0	0	0	0	1	0	0	0.020
0.72 ug/ml	A	6.4	50	2	0	0	0	0	1	0	0	0.020
	B	5.8	50	0	0	0	0	0	0	0	0	0.000
1.44 ug/ml	A	3.8	50	0	0	0	0	0	0	0	0	0.000
	B	4.2	50	6	0	2	0	1	0	0	0	0.060
TEM,	A	5.0	50	34	1	10	2	15	0	1	0	0.560
0.25 ug/ml	B	5.0	50	18	0	4	1	7	0	0	0	0.240

[1]CHO cells were treated for 12 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 4
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR242511 TARTRATE IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
					14 Hour Harvest							
Untreated cells	A	6.6	50	0	0	0	0	0	0	0	0	0.000
	B	7.0	50	0	0	0	0	0	0	0	0	0.000
DMSO	A	6.2	50	0	0	0	0	0	0	0	0	0.000
	B	5.6	50	2	0	0	0	0	0	1	0	0.020
WR242511 Tartrate												
0.6 ug/ml	A	6.0	50	0	0	0	0	0	0	0	0	0.000
	B	6.6	50	0	0	0	0	0	0	0	0	0.000
1.2 ug/ml	A	5.8	50	0	0	0	0	0	0	0	0	0.000
	B	4.8	50	2	0	1	0	0	0	0	0	0.020
2.4 ug/ml	A	4.4	50	4	0	3	0	0	0	0	0	0.060
	B	3.8	50	4	0	2	0	1	0	0	0	0.060
4.8 ug/ml	A	2.2	50	6	0	3	0	0	0	0	0	0.060
	B	2.6	50	2	0	1	0	0	0	0	0	0.020
CP,	A	4.6	50	6	0	0	1	1	1	0	0	0.060
25 ug/ml	B	5.8	50	14	0	2	0	7	0	0	0	0.180

[1]CHO cells were treated for 2 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 5
SUMMARY

Treatment	S-9 Activation	Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	14	5.2	100	0.020 ± 0.141	2
DMSO	-	14	6.9	100	0.010 ± 0.100	1
WR242511 Tartrate						
0.18 ug/ml	-	14	5.6	100	0.010 ± 0.100	1
0.36 ug/ml	-	14	4.7	100	0.020 ± 0.141	2
0.72 ug/ml	-	14	6.1	100	0.010 ± 0.100	1
1.44 ug/ml	-	14	4.0	100	0.030 ± 0.171	3
TEM, 0.25 ug/m	-	14	5.0	100	0.400 ± 0.816	26
Untreated	+	14	6.8	100	0.000 ± 0.000	0
DMSO	+	14	5.9	100	0.010 ± 0.100	1
WR242511 Tartrate						
0.6 ug/ml	+	14	6.3	100	0.000 ± 0.000	0
1.2 ug/ml	+	14	5.3	100	0.010 ± 0.100	1
2.4 ug/ml	+	14	4.1	100	0.060 ± 0.312	4
4.8 ug/ml	+	14	2.4	100	0.040 ± 0.197	4
CP, 25 ug/ml	+	14	5.2	100	0.120 ± 0.383	10

[1]Severely damaged cells were counted as 10 aberrations.
[2]*, p≤0.05; **, p≤0.01; Fisher's exact test.

REFERENCES

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Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

APPENDIX

Study Protocol

APPROVED
QC 6-3-93

MA Study Number: TD146.337

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: **TOXICOLOGY RESEARCH LABORATORY**

2.2 Address: University of Illinois at Chicago
Department of Pharmacology Box 6998
Chicago, Il 60680

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T.

2.4 Sponsor Project #: UIC-7. This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR242511 Tartrate**

Storage: -20 to -15°C
Ambient humidity conditions
Protect from light

3.2 Controls:

Solvent:	Dimethysulfoxide (DMSO)
Negative:	Untreated Cells
Positive:	Cyclophosphamide (CP) & Triethylenemelamine (TEM)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Division of Genetic and Cellular Toxicology Microbiological Associates, Inc.
4.2	Address:	9900 Blackwell Road Rockville, MD 20850
4.3	Study Director:	Donald L. Putman, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 7-6-93

5.2 Proposed Experimental Completion Date: 9-28-93

5.3 Proposed Report Date: 10-2-93

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing CHO cells to a minimum of four concentrations of the test article as well as negative and positive controls in duplicate cultures for 8 hours or up to 18 hours in the event of cell cycle delay in the absence of an S-9 activation system and for 2 hours in the presence of an S-9 reaction mixture. The dividing cells will be arrested in metaphase at a time after initiation of treatment selected to yield first-division metaphase cells and harvested for microscopic evaluation of chromosome aberrations. The mutagenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon mitotic inhibition after treatment. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the limit of solubility but not to exceed 5 mg/ml. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition in treatment medium will also

be measured. Cells seeded 16-24 hours earlier will be exposed for 6-8 hours in the absence of S-9 and for 2 hours in the presence of S-9. Following a 24 hour post-treatment growth period in medium containing 0.01mM 5-bromo-2'-deoxyuridine (BrdU), with Colcemid (0.1 ug/ml) present for the last 2 hours, the cells will be harvested by trypsinization. Metaphase preparations will be made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides will be evaluated for the percentage of first-, second- and third-plus-subsequent-division metaphase cells per 100 cells scored for determination of the test article effect on cell cycle kinetics. The percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group.

Whenever possible, the high dose will be selected to give at least 50% toxicity (mitotic inhibition). At least three additional dose levels will be included in the assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, if excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if osmolality of the treatment medium is excessive, the assay will be set up at the highest concentration meeting protocol requirements. The cell cycle kinetics study will be used to determine the optimum harvest time for the cytogenetic study.

7.2 Frequency and Route of Administration

Target cells will be treated for 8-18 hours depending upon cell cycle delay in the absence of S-9 and for 2 hours in the presence of S-9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of exposure of mammalian cells in culture to various concentrations of chemical.

7.3 Activation System

Aroclor 1254-induced rat liver S-9 (prepared under ACUC approved application Permit No. 4) will be used as the metabolic activation system. The S-9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with a cofactor pool to contain 1.4 mg NADP, 2.7 mg isocitric acid and 15 ul S-9 per ml serum-free McCoy's 5A medium.

7.4 Controls

7.4.1 Untreated Control

Untreated cells will be used as the untreated control.

7.4.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. The solvents compatible with this test system include water or medium, dimethylsulfoxide, acetone and ethanol. For solvents other than medium, the final concentration in treatment medium will not exceed 1%.

7.4.3 Positive Controls

Triethylenemelamine will be used at a concentration within 0.25-1 ug/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 25-100 ug/ml as the positive control in the S-9 activated study.

7.5 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 ug streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air for 16-24 hours.

7.6 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.7 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated study or 5 ml S-9 reaction mixture for the S-9 activated study, to which will be added 50 ul of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if medium is used as the solvent. An untreated control consisting of cells in complete medium or S-9 reaction mixture will also be included.

In the non-activated study, the cells will be exposed for 8 hours at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. Exposure will be extended to up to 18 hours in those dose levels with considerable cell cycle delay. Exposure will continue until Colcemid treatment at which time the treatment medium will be removed to avoid interference with cell collection and fixation.

In the S-9 activated study, the cells will be exposed for 2 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the exposure period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

7.8 Collection of Metaphase Cells

Cells will be collected approximately 10 hours after initiation of treatment or at a later time selected to represent the first division metaphase after initiation of treatment if the test article causes considerable cell cycle delay. Cell fixation will not extend beyond 20 hours after initiation of treatment. Two hours prior to harvest, Colcemid will be added to the cultures at a final concentration of 0.1 ug/ml.

Cells will be harvested by trypsinization, collected by centrifugation, swollen with 0.075 M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 0-6°C. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. One to two slides will be prepared from each treatment flask. The slides will be stained with Giemsa and permanently mounted.

7.9 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads from each dose level (50 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented for the toxicity and aberration study. The number and types of aberrations found will be presented for each treatment group. The percentage of structurally damaged cells in the total population of cells examined will be calculated for each group. The mean aberrations per cell will also be calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data. Data will be presented in tabular form for the relative toxic effects of treatment, the number and types of aberrations per cell and the frequency of cells with aberrations.

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc. in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill EPA and OECD test guidelines.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes
If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? yes

If so, indicate which doses and the volume that will be required. _____ high only;
_____ high and low only;
___X___ all;
volume required 10 ml (if possible).

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

14.0 APPROVAL

Barry S Levine 12/3/93
SPONSOR REPRESENTATIVE DATE

Barry S Levine
(Print or Type Name)

[illegible] 12-8-92
U.S. ARMY CONTRACTING OFFICER'S REPRESENTATIVE DATE

GEORGE J. SCHIEFERSTEIN.
(Print or Type Name)

[illegible] 6-3-93
STUDY DIRECTOR DATE